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DOCKET NO.: CHIR-0157 (0316.006) **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:
Covacci *et al.*

Serial No.: 09/360,685 **Group Art Unit:** 1645

Filing Date: July 26, 1999 **Examiner:** S. Devi

For: HELICOBACTER PYLORI CAI ANTIGEN PROTEINS USEFUL FOR VACCINES AND DIAGNOSTICS

DATE OF DEPOSIT: January 8, 2002

I HEREBY CERTIFY THAT THIS PAPER IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL, POSTAGE PREPAID ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, DC 20231.

TYPED NAME: Robin S. Quartin
REGISTRATION NO.: 45,028

Assistant Commissioner for Patents
Washington DC 20231

Sir:

COMMUNICATION

Enclosed is the original, executed Declaration Pursuant to 37 C.F.R. §1.132 of Antonio Covacci with Exhibits A-C.

An unexecuted copy of this document was attached to our Request for Continued Examination (RCE) which was filed with the United States Patent and Trademark Office on July 30, 2001.

Respectfully submitted,

Robin S. Quartin
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Date: January 8, 2002

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DOCKET NO.:CHIR-0157 (0316.005) PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **Covacci *et al.***

Serial No.: **09/360,685** Group Art Unit: **1638**

Filed: **July 26, 1999** Examiner: **P. Bui**

For: **HELICOBACTER PYLORI CAI ANTIGEN PROTEINS USEFUL FOR VACCINES AND DIAGNOSTICS**

I, Robin S. Quartin, Registration No. **45,028** certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 on

January 8, 2002

Robin S. Quartin Reg. No. **45,028**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

I, Antonio Covacci, do hereby declare as follows:

1. I am the Senior Research Director of the Bioinformatics Unit at Chiron SpA, in Siena, Italy.

2. I am a medical doctor (M.D.) with 17 years of experience in bacterial pathogenesis. My curriculum vitae is attached hereto as Exhibit A.

3. I am a co-inventor of the subject matter of U.S. application serial number 09/360,685, filed July 26, 1999, entitled "*Helicobacter pylori* CAI antigen proteins useful for vaccines and

diagnostics" ("'685 application"). The '685 application claims priority to PCT applications PCT/EP93/00158 (filed January 25, 1993) and PCT/EP93/00472 (filed March 2, 1993), both of which claim priority benefit of Italian application Serial No. FI92A000052 (filed March 2, 1992).

4. The invention provides polypeptides of *Helicobacter pylori* cytotoxin associated immunodominant antigen (CagA)[1], for use, among others, in vaccines. The molecular weight of CagA, as reported in the specification for strain CCUG 17874, is 128 kDa.

5. I have read the Official Action dated October 24, 2000 ("Action").

6. In the Action, the Examiner rejected claims 60 and 62, which are directed to methods of preparing vaccines comprising recombinant CagA, as obvious over Cover *et al.* (1990) Infect. Immun., 58:603-610 ("Cover"). The Examiner's argument is based upon the Examiner's assumption that Cover had purified CagA and that Cover had immunized rabbits with a purified CagA protein. I respectfully disagree that the claims are obvious over Cover, and that the CagA protein was known.

7. The problem of purifying and characterizing the CagA protein was very complex at the time of the earliest priority date of the '685 application. The protein had not been purified at the priority date of March 2, 1992. All that was known in the art about CagA was (1) it had a high apparent molecular weight (120, 128, or 130 kDa; specification at page 2, lines 24 - 31), and (2) it was associated with *H. pylori* strains that were cytotoxic (specification at page 6, lines 20 - 26).

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[1]In the specification of the '685 application, *H. pylori* cytotoxin associated immunodominant antigen is referred to as "CAI." However, the current terminology used for this protein is the "cytotoxin-associated gene A" or "CagA" antigen.

8. The state of the art regarding the CagA protein at the time of the earliest priority date of the '685 application was remarkably sparse. No amino acid sequence information was available (specification at page 2, lines 35 - 36). No one had purified, microsequenced or even analyzed the amino acid composition of a *H. pylori* cytotoxin associated immunodominant antigen having a molecular weight in the range of 128 kDa. Today we can attribute this to the highly labile and unstable nature of CagA. Indeed, to date, there is no report of production of a crystal of CagA protein.

9. Cover added nothing to the sparse body of knowledge regarding CagA. Cover only described a 128 kDa band on an immunoblot. Cover could not identify a corresponding band by the highly sensitive technique of silver staining, and took no steps to purify or sequence any protein corresponding to the 128 kDa immunoblot band.

10. We at Chiron ultimately succeeded in purifying the CagA protein. Our success in purification of the CagA protein contributed to our success in cloning the CagA gene. Our method of culturing the *H. pylori* and preparation of material for gel electrophoresis were optimized to yield stable, purified CagA protein. We prepared liquid cultures of *H. pylori* in Brucella broth containing fetal bovine serum and cyclodextrin (specification at page 48, lines 16 - 20). Cover did not report carrying out this procedure.

11. Pelleted *H. pylori* cells from the foregoing were treated with 6M guanidine, loaded directly onto acrylamide gels and electrophoresed (specification at page 50, lines 11 - 12). Cover did not report following this protocol.

12. The band representing the CagA protein, which was visualized by Coomassie staining, was isolated from the gel (specification at page 50, lines 25 - 29). No such steps were reported to be taken by Cover.

13. The CagA protein, thus prepared, was used to immunize mice to generate polyclonal antibodies to CagA (specification at page 50, lines 29 - 31). Cover did not report carrying out such a procedure. These polyclonal antibodies were used in the screening of a library of *H. pylori* λgt11 expression clones, detecting one positive clone in every 3000 (specification at page 51, lines 3 - 4 and 6 - 7). No such screening was reported as carried out by Cover.

14. It was very difficult to make genomic libraries of *H. pylori* at the time of the earliest priority date of the '685 application. In addition to the λgt11 expression library, from which various expression clones of small inserts were identified, our strategy for cloning the entire CagA gene included efforts to generate a complete genomic *H. pylori* library containing large fragments of *H. pylori* DNA. Our initial attempts, using vectors that accommodate large DNA inserts such as EMBL4 and λDASH, encountered the same difficulties of others in the art trying to clone *H. pylori* DNA (see specification at page 49, lines 17 - 20). The difficulties encountered by others attempting to clone *H. pylori* DNA are described at pages 47 - 48 in Taylor (1992) Annu. Rev. Microbiol., 46:35-64 (attached hereto as Exhibit B).

15. Furthermore, S. Nooria, a staff scientist at CLONTECH Laboratories, Inc. ("CLONTECH"), described CLONTECH's difficulties in attempting to clone *H. pylori* DNA, in a letter dated July 27, 1992 (attached hereto as Exhibit C). We had provided *H. pylori* DNA to CLONTECH about 6 months earlier, and asked them to try to carry out the cloning. Although CLONTECH tried several cloning systems (the EMBL3 vector and a related Sp6/T7-containing vector) and bacterial strains, they failed to make a *H. pylori* library.

16. We were ultimately successful in generating a partial genomic plasmid library by making two important choices. We chose to use (1) the pBluescript vector to make the plasmid library, and (2) the *E. coli* strain DH 10B to propagate the library (specification at page 49, lines 21 - 25). *H. pylori* has a highly AT-rich genome, and the region of the *H. pylori* genome that contains the CagA gene is extremely unstable in *E. coli*. The vector system and particular strain of *E. coli* chosen by us are particularly tolerant to AT-rich genomes, contributing to our success. From this library, we were able to isolate clones such as B1 (specification at page 51, lines 14 - 15, and Figure 3), which contained the 3' half of the CagA gene. The clones identified from the λgt11 library and the pBluescript clone B1 were used to determine the complete nucleotide sequence for the CagA gene.

17. I declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Antonio Covacci, M.D. Date 12/21/2001

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1990-1991	Postdoctoral scientist, Hormone Research Institute, UCSF
1988	Practicing Physician Abilitation
1977-1988	Medical Degree, University of Florence School of Medicine, Florence- Italy
1971-1976	Scientific Lyceum " P. Ruffini", Viterbo- Italy

Awards and Honors:

1996	Italian Chemical Society Award
1994	Lepetit Award
1990-1991	American Diabetes Society Fellowship Award
1988	Graduated *Summa cum laude* with Special Mention
1984-1988	Junior Fellow of Sclavo Research Center
1976	Diploma *Magna cum laude*
1971-1976	Gold Medal " G. Kirner Institute"

Research Experience:

2000	Senior Research Director, Head of Bioinformatics Unit, Chiron SpA
1998- 2000	Principal scientist
1994 -1998	Project Leader Department of Molecular Biology IRIS/Chiron, Siena
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Publications

Del Giudice G, Covacci A, Telford JL, Montecucco C, Rappuoli R. The design of vaccines against helicobacter pylori and their development. Annu Rev Immunol. 2001;19:523-63.

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GENETICS OF *CAMPYLOBACTER* AND *HELICOBACTER*

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KEY WORDS: genetic transformation, bacterial plasmids, cloning vectors, genome mapping, antibiotic resistance

CONTENTS

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Abstract

This article reviews the current state of genetic analysis of *Campylobacter* and *Helicobacter*. Chromosomal genes cloned from *Campylobacter* and *Helicobacter* species are listed along with the method used to identify the cloned gene. *Campylobacter* plasmid genes that have been cloned and expressed in *Escherichia coli* and that specify resistance to tetracycline, kanamycin, or chloramphenicol are presented.

This review also examines our current knowledge of genetic exchange in *Campylobacter*, including conjugative plasmid transfer, natural transformation, electrotransformation, and bacteriophage transduction. In *Helicobacter*, natural transformation has been described and both plasmids and bacteriophages have been observed. Plasmid cloning vectors have been constructed for *Campylobacter*. Available vectors are discussed and restriction maps of some useful vectors that we have constructed are included.

The genome sizes of *C. jejuni* and *C. coli* are approximately 1.7 megabases (Mb), whereas the genome size of *H. pylori* ranges from 1.60 to 1.73 Mb. The positions of various genes on the *C. jejuni* and *C. coli* genome maps have been determined using both homologous and heterologous DNA probes. Genomic maps of these organisms are presented.

INTRODUCTION

This review deals with genetic developments in the genera *Campylobacter* and *Helicobacter*. Bacteria in these genera have a spiral or S-shaped morphology and are 0.5 to 8.0 μm long and 0.2 to 0.5 μm wide. They are gram negative and microaerophilic, with a G+C content that varies from 28 to 44 mol%. Species of *Campylobacter* have a single polar unsheathed flagellum at one or sometimes both ends of the cell, whereas species of *Helicobacter* have four to six sheathed flagella at one or sometimes both poles. The decision to transfer *Campylobacter pylori* (formerly *C. pyloridis*) to the new genus *Helicobacter* (41) was based on ribosomal RNA sequencing (118), fatty acid profiles, biochemical reactions, and morphological characteristics. The taxonomic position of several members of the *Campylobacter* genus is currently in a state of flux (149).

Campylobacter species are pathogens or commensals in a wide range of animal species. *Campylobacter jejuni* is a common cause of human diarrheal illness (125), and several other *Campylobacter* species, *C. coli*, *C. fetus* subsp. *fetus*, *C. hyointestinalis*, *C. lari*, and *C. upsaliensis* (46), can also cause similar disease manifestations. In contrast, *Helicobacter pylori* is frequently isolated or observed in preparations from gastric biopsies of patients with gastritis and/or ulcers (82, 83). Other recently named *Helicobacter* species include *H. mustelae*, *H. felis*, and *H. nemestrinae* isolated, respectively, from stomach tissue of ferrets (37), cats (107), and the pigtailed macaque (13).

The considerable recent interest in both *Campylobacter* and *Helicobacter* has resulted in several excellent review articles about these organisms. For information on the association of gastric disease and *H. pylori*, the reader is referred to other reviews (8, 16, 31, 32, 42, 44, 110). Recent reviews of *Campylobacter* have stressed taxonomy (109), epidemiology (12), association with human disease (46), antibiotic resistance (133) and pathophysiology, and early genetic studies (152).

GENES CLONED FROM *CAMPYLOBACTER* AND *HELICOBACTER*

Campylobacter *House-Keeping Genes*

Table 1 lists chromosomal genes that have been cloned from *Campylobacter*. Of those that have been cloned from *C. jejuni* or *C. coli* and successfully expressed in *Escherichia coli*, all can be classified as general house-keeping genes. Housekeeping genes are those that appear to be highly conserved across species boundaries, and encode similar functions required for the maintenance and growth of many different bacterial species, e.g. those required for amino acid biosynthesis.

In 1985, Lee and coworkers (72) reported that they had cloned in *E. coli* two genes from *C. jejuni* required for proline biosynthesis by selection for complementation in a *proAproB* mutant of *E. coli*. Although the DNA sequence of the DNA fragment from the *C. jejuni* gene library has not been determined (P. Guerry, personal communication), a similar strategy has been used to clone the *proA* gene and determine its sequence (V. L. Chan, personal communcation). Investigators have used this complementation procedure to clone several genes from amino acid pathways (see Table 1). These strategies have probably been successful because they rely on strong selective pressure: the requirement for growth of *E. coli* in absence of a particular amino acid to maintain the *Campylobacter* gene(s). Completion of the DNA sequences of these genes is important for comparison with homologous genes found in other species and to determine if they are stable in *E. coli* in the absence of selective pressure. Answers to these questions may help explain why difficulties have been encountered in cloning certain *Campylobacter* genes (see below).

Other house-keeping genes cloned from *C. jejuni* include 16S and 23S ribosomal RNA genes (59, 115), which were identified by hybridization of ribosomal RNA from *C. jejuni*. Two transfer RNA genes (those for alanine and leucine) were also cloned and were identified by their proximity to a 16S rRNA gene (116). The DNA sequences of these tRNA genes have been determined.

Table 1 Genes cloned from *Campylobacter* species and *Helicobacter* species

Microorganism	Gene cloned[a]	Cloning strategy[b]	Location	DNA sequence published	Reference
C. jejuni	γ Glutamyl kinase *(proA)*	Reverse genetics[c]	Chromosome	No	72; V. L. Chan[d]
C. jejuni	γ Glutamyl phosphate reductase *(proB)*	Reverse genetics[c]	Chromosome	No	72
C. jejuni	Serine hydroxylmethyltransferase *(glyA)*	Reverse genetics[c]	Chromosome	Yes	21, 22
C. jejuni	β-Isopropylmalate (IPM), dehydrogenase *(leuB)*, IPM isomerases *(leuC, leuD)*	Reverse genetics[c]	Chromosome	No	A. Labigne[d]
C. jejuni	Acetylornithinase *(argE)*	Reverse genetics[c]	Chromosome	No	D. E. Taylor & M. Bussiere[e]
C. jejuni	Arginosuccinase *(argH)*	Reverse genetics[c]	Chromosome	No	V. L. Chan[d]
C. jejuni	Flagellin *(flaAflaB)*	λgt11 + antibody[f]	Chromosome	Yes	98, 99
C. jejuni	Flagellin *(flaA)*	PCR[g]	Chromosome	Yes	35
C. coli	Flagellin *(flaAflaB)*	Oligonucleotide probe[h]	Chromosome	Yes	48, 77
C. jejuni	Major outer membrane protein (MOMP)	λgt11 + antibody[f]	Chromosome	No[i,j]	138
C. jejuni	Ribosomal RNA: 16S, 23S	Hybridization of rRNA	Chromosome	No[j]	59, 115
C. jejuni	tRNA (Ala), tRNA (Leu)	Located next to 16S rRNA gene	Chromosome	Yes	116
C. fetus	Surface-array protein *(sapA)*	λgt11 + antibody[f]	Chromosome	Yes	9
C. coli	Chloramphenicol acetyltransferase *(cat)*	Direct selection[k]	Plasmid	Yes	120, 155
CLO[l]	Aminoglycoside phosphotransferase *(aphA-1)*	Direct selection[k]	Chromosome	Yes	104
C. coli	Aminoglycoside phosphotransferase *(aphA-3)*	Direct selection[k]	Plasmid	Yes	148
C. coli	Aminoglycoside phosphotransferase *(aphA-7)*	Direct selection[k]	Plasmid	Yes	144
C. jejuni	Tetracycline resistance *(tetO)*	Direct selection[k]	Plasmid	Yes	128
H. pylori	Tetracycline resistance *(tetO)*	Direct selection[k]	Plasmid	Yes	81, 131, 140
H. pylori	Urease *(ureA, ureB)*	λgt11 + antibody[f]	Chromosome	Yes	28, 29
	Urease *(ureA, ureB, ureC, ureD)* *(ureE, ureF, ureG, ureH)*	Urease production[m]	Chromosome	Yes	68
H. pylori	26,000-Dalton protein[n]	Oligonucleotide probe[h]	Chromosome	No	29a
				Yes	103

[a] The complete name of the genetic property that has been cloned is given. The genotypic designation, where available, is included in parentheses.
[b] Refers to the strategy used to obtain the gene in question.
[c] Reverse genetics refers to complementation of an *E. coli* mutant by cloned gene(s) from *Campylobacter* species, e.g. complementation of *proA* and *proB* mutations.
[d] Personal communication.
[e] In preparation.
[f] Refers to the construction of gene libraries using phage λgt11 and subsequent screening with antibody to the protein of interest (see 55).
[g] PCR, sequence determined using polymerase chain reaction and oligonucleotide primers.
[h] Selected by hybridization of an oligonucleotide probe based on the amino acid sequence of the protein of interest.
[i] Complete open reading frame of MOMP has not yet been cloned.
[j] Partial DNA sequence of MOMP available (K. Hiratsuka & D. E. Taylor, unpublished data); sequences of 16S and 23S rDNA genes available from Dr. S. Cohen, Gene Track Inc., Boston, MA.
[k] Direct selection for expression of an antibiotic-resistance phenotype after cloning in *E. coli*.
[l] CLO, *Campylobacter*-like organism.
[m] Genes *(ureA, ureB, ureC, ureD)* were identified by transient urease production in *C. jejuni* (68); genes *(ureE, ureF, ureG* and *ureH)* are required for expression in *E. coli* (29a).
[n] Identity of the 26-kDa protein is as yet unknown.

Campylobacter *Virulence Genes*

C. jejuni and *C. coli* cause much gastrointestinal morbidity. Therefore, genetic studies have focused on structural proteins and other products believed to play a role in pathogenesis. These studies have mainly examined flagella of *C. jejuni* and *C. coli;* however, an enterotoxin and major outer membrane protein of *C. jejuni*, as well as a surface protein of *C. fetus fetus*, have also been investigated.

FLAGELLAR GENES Flagella play an important role in virulence because the bacterium needs them to colonize the intestines. Aflagellate mutants and nonmotile strains cannot colonize in animal models (1, 89, 96). In addition, the flagella are highly immunogenic and patients produce antibody to flagella soon after infection (7, 92, 158).

The genetics of flagellar production have been investigated extensively in both *C. jejuni* 81116 (98, 99, 156) and *C. coli* VC167 (47–49, 77, 146) and to a lesser extent in *C. jejuni* IN1 (34). *C. jejuni* 81116 and *C. coli* VC167 contain two copies of the flagellin genes, designated *flaA* and *flaB*, located adjacent to one another in a head-to-tail configuration (48, 99). In *C. jejuni* 81116, both genes comprise 1731 base pairs that are 95% identical. Primer extension studies demonstrated that *flaA* mRNA is transcribed from a σ^{28} promotor in *C. jejuni* 81116 (97). In *E. coli*, this promoter transcribes genes involved in chemotaxis, motility, and flagella function (2). The *flaB* gene lacks any recognizable promoter sequence, and initial studies suggested that it is not transcribed (97). However, more recent work suggests that a very low level of transcription from *flaB* may occur (156). In *C. coli* VC167, both *flaA* and *flaB* genes possess promoters. Although the former is a typical σ^{28} promotor, the latter appears to resemble a (*nif*) σ^{54} promoter (47). This promotor is usually activated in response to nitrogen starvation (67). The *flaB* gene is expressed at low levels in *C. coli* VC167 (47).

The calculated molecular weights of flagellin A and B estimated from the *flaA* and *flaB* genes of *C. jejuni* 81116 are 59,538 and 59,909, respectively. However, the observed molecular weight of the flagellin in polyacrylamide gels is 62,000, although sometimes a second flagellar protein of 60,000 is also observed (95). These two observed flagellins of different sizes do not represent products of the *flaA* and *flaB* genes, because a monoclonal antibody that recognizes both proteins reacted with expressed *flaA* fragments but not with homologous *flaB* fragments (100). These size differences may result from posttranslational modification of amino acid residues within the flagellar protein (99).

The availability of the deduced amino acid sequences derived from DNA sequencing of three *flaA* genes enables the identification of common and variable regions within the flagellin proteins. Two common regions consisting

of the 170 amino acid N-terminal region (C1) and the 100 amino acid C-terminal region (C2) exhibit 94 and 96% identity, respectively. A variable region, V1, occupies the middle of the flagellin. Several areas within the V1 region are predicted to be surface-exposed and probably correspond to areas with surface epitopes (35).

Deletion mutant analysis utilizing a gene replacement technique (69) showed that *flaA*$^+$*flaB*$^-$ derivatives of both *C. jejuni* 81116 and *C. coli* VC167 have normal flagella, whereas *flaA*$^-$*flaB*$^-$ mutants are aflagellate (47, 156). Mutants of *flaA*$^-$*flaB*$^+$ type in both strains had short truncated filaments and much reduced motility compared with wild-type. Mutants of VC167 that are *flaA*$^+$*flaB*$^-$ were slightly less motile than wild-type cells, although they produced a flagellar filament indistinguishable in length from wild-type (47). Antiserum specific for the *flaB* gene product reacted sparsely along the entire length of the filament in *flaA*$^+$*flaB*$^+$ cells. These results suggest that the flagellar filament of *C. coli* VC167 is composed of both *flaA* and *flaB* gene products and that the *flaB* gene product constitutes less than 20% of the wild-type filament (47). Such an organization with the *flaB* subunit intertwined among *flaA* subunits is reminiscent of that seen in the complex flagellar filaments of *Rhizobium meliloti* (111), *Caulobacter crescentus* (88), and also apparently in *H. pylori* (65). In contrast, the *C. jejuni* 81116 flagellum contains only the *flaA* gene product (99, 100, 156).

Both *flaA* and *flaB* genes appear to be present in most *Campylobacter* strains examined using DNA hybridization analysis (146). These genes apparently have been maintained throughout evolution and presumably confer some selective advantage on the host bacterium. The data of Guerry et al (47) suggest that the *flaB* gene product confers increased motility on the flagella, but this is apparently not the case with all strains. The two copies of *fla* genes may be maintained within the *Campylobacter* genome to provide a duplicate in case the expression copy undergoes a mutation or deletion event rendering it nonfunctional. Mistakes in the expression copy could be corrected by a recombinational event within the alternate copy. In addition, genetic exchange among flagellar genes could involve natural transformation (see below) in which DNA from lysed cells in the population may be taken up. Such a process could play an important role in generating antigenic diversity in *Campylobacter* species.

PHASE VARIATION Flagellar expression in *Campylobacter* spp. is subject to both phase and antigenic variation (47, 49, 51, 76). Phase variation refers to the ability of some strains of *Campylobacter* to switch on and off flagellar production. Caldwell et al (19) showed that *C. jejuni* 81116 cells undergo a bidirectional transition between flagellated and aflagellated phenotypes. The Fla$^+$ → Fla$^-$ transition occured at rates approximately 3×10^{-3} per cell per

generation whereas the $Fla^- \rightarrow Fla^+$ transition occurred at the rate of approximately 4×10^{-7} per cell per generation. Passage through the rabbit intestine selected for emergence of the Fla^+ phenotype. Although many strains of *C. jejuni* and *C. coli* undergo phase variation (158, 159) and aflagellate variants are simple to select in vitro, the genetic events involved are far from clear. Presumably some rearrangement could occur in the upstream region of *flaA* that turns off flagellar synthesis. However, this event must be reversible because Fla^+ variants can be selected either by passage through an animal model or in the laboratory. Other alternatives could include a repressor that turns off flagellar synthesis or an activator required to turn it on. The situation may be similar to that seen in other pathogens, such as *Bordetella pertussis* (64) and *Vibrio cholerae* (87), that have coordinate regulation of virulence gene expression. Research may yet show that other virulence traits in *Campylobacter* spp. are controlled at the genetic level by such a system.

ANTIGENIC VARIATION Antigenic variation refers to the ability of *Campylobacter* species to reversibly express flagella of different antigenic specificities (51). In *C. coli* VC167, antigenic variation corresponds to the production of two flagellins of different molecular weights, 61,500 (T1) and 59,500 (T2) (47, 51). Isoelectric focusing of purified flagellar filaments from several *C. jejuni* serotypes also showed multiple charged flagellins (91). These size and charge differences may result from posttranslational modification of amino acid residues within the flagellar protein(s) (76, 77). Guerry et al (48, 49) showed that *C. coli* VC167 undergoes a DNA rearrangement associated with the flagellar antigenic variation. The rearrangements were detected using a 700–base pair probe from VC167 that contains homology to members of the *Enterobacteriaceae*. Further studies suggest that the flagellar variation was associated with an uncharacterized rearrangement adjacent to a 23S rDNA locus (P. Guerry, personal communication). The molecular events underlying antigenic variation are complex and unclear.

EXPRESSION OF *CAMPYLOBACTER* FLAGELLIN GENES IN *E. COLI* Molecular analysis of the flagellin genes has been hampered by the inability of *E. coli* to express detectable levels of the proteins. The *flaA* genes from both *C. jejuni* 81116 and *C. coli* VC167 possess a strong σ^{28} type promotor, and fusion of the *flaA* promotor to a promotorless chloramphenicol acetyltransferase gene has demonstrated that the promotor can function in *E. coli* (48). Other reasons for lack of expression in *E. coli* include codon utilization differences (124) and lack of posttranslational modification of amino acids in *E. coli*. Although *flaA* genes specify mRNA containing suboptimal codons for peptide synthesis in *E. coli* (48), this is probably not the major factor in lack of expression. Fusion of the *flaA* and *flaB* proteins with the *cro-lacZ* protein was obtained using the pEX series of vectors (100). In this system, the higher proportion of rare codons in *C. jejuni* DNA compared with *E. coli* did not appear to be a limiting factor for expression. The advantage of this fusion protein expression system is that the fusion proteins precipitate inside the cell and are protected from proteolysis.

ENTEROTOXIN GENE Some strains of *C. jejuni* produce an enterotoxin (56, 61–63, 85, 119). Klipstein et al (62) found a correlation between enterotoxin production by strains of *Campylobacter* and watery diarrhea; however, many strains of *C. jejuni* and *C. coli* isolated from stool specimens do not appear to produce enterotoxin (38). Studies with DNA probes for cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) genes could not demonstrate homology at the molecular level between *C. jejuni* and *C. coli* DNA and CT or LT genes (5, 102, 152). *Campylobacter* enterotoxin genes are chromosomal and not plasmid mediated (141). Using an oligonucleotide probe similar to the coding region for a postulated ganglioside GM1-binding site on the *toxB* gene from *Vibrio cholerae* and the *eltB* gene from *E. coli*, Calva et al located *C. jejuni* homologous chromosomal sequences (20). The oligonucleotide hybridized to a *Sau*3A digest of total DNA of all tested *C. jejuni* isolates. Because not all *C. jejuni* strains produced the enterotoxin, the authors concluded that some of the enterotoxin genes are probably inactive.

Work on enterotoxins has been complicated by disagreements arising partly from the use of different cell lines, different culture conditions, and different methods of observation, so it is important to clone the putative enterotoxin gene both for DNA sequencing and expression studies. However, Calva et al report difficulties in cloning and maintaining this and other *C. jejuni* sequences in *E. coli* hosts (20). More recently, increased stability of *C. jejuni* DNA recombinants was obtained by cloning in a *mutL* derivative (deficient in methyl-directed DNA repair) (E. Calva, personal communication).

MAJOR OUTER MEMBRANE PROTEIN Fusion proteins containing a portion of the major outer membrane protein (MOMP), were obtained using a polyclonal antibody directed against the MOMP from *C. jejuni* UA580 to screen λgt11 libraries. Two different DNA fragments of 147 base pairs (bp) and 1845 bp were identified (138). One fragment hybridized only with *C. jejuni* DNA and can be used as a *C. jejuni*–specific probe, whereas the other hybridized with all *C. jejuni* and *C. coli* strains tested, as well as with some *C. lari* strains (139). DNA sequencing (K. Hiratsuka & D. E. Taylor, unpublished data) demonstrated that both of these fragments lacked the complete open reading frame for the MOMP believed to be a porin (54). Although porins are not strictly virulence determinants, the MOMPs in *Campylobacter* spp. act as major immunogens and may be involved in the uptake and

exclusion of various antibiotics (53, 106). Work is in progress to complete the sequence of these putative MOMP genes and to compare the deduced protein sequences to porins of other species.

SURFACE-ARRAY PROTEIN OF *C. FETUS* Surface arrays (S-layers), which consist of regularly arranged protein subunits that are self-assembling and form two-dimensional paracrystalline arrays of protein monomers, have been observed in *C. fetus* subsp. *fetus* (10, 36). Wild-type *C. fetus* strains that possess S-layers, but not spontaneous mutants that lack them, resist killing by normal and immune serum and resist phagocytosis because of defective binding of host C3b to bacterial cell surfaces (11).

Polyclonal antibody to surface-array proteins (SAP) of *C. fetus* with molecular weights of 97,000–149,000 were used to select clones from a library of λgt11 into which *C. fetus* chromosomal fragments of 1.0–6.5 kilobases (kb) had been ligated (9). A clone with a 4.0-kb insert was subcloned in pUC9, and expression of a protein of 98,000 Daltons was obtained in *E. coli*. The protein was not fused with β-galactosidase nor was expression inducible by isopropyl-thiogalactoside (IPTG). These studies failed to identify a promoter sequence, and expression was assumed to depend on a flanking promotor present within the vector. The complete sequence of the *C. fetus sapA* gene was determined; it encodes a 933–amino acid polypeptide with a calculated molecular weight of 96,758. Because the first 20 amino acids matched exactly those determined from N-terminal sequencing of the SAP protein, this polypeptide is apparently secreted without a leader sequence. The *C. fetus* SAP contains a small but distinctive region within a hydrophobic region that is homologous with other S-layer proteins from *E. coli*, *Klebsiella pneumoniae*, and *Yersinia* and *Leishmania* species. There is little overall homology among either primary or secondary structures of the S-layer proteins for which structural genes have been cloned, as would be expected because S-layer proteins appear to be biochemically diverse. Further study of the *C. fetus* SAP protein is eagerly awaited, because this protein appears to contain some interesting functional domains including sites for C3b interaction, procoagulant activity, and calcium-binding.

Campylobacter *Antibiotic Resistance Genes*

Genes encoding resistance to three different antibiotics in *Campylobacter* species have been cloned and sequenced (Table 1). Resistance to the antibiotics kanamycin, tetracycline, and chloramphenicol is usually plasmid mediated, and some plasmids carry more than one antibiotic-resistance determinant (133). Several of these antibiotic-resistance determinants are believed to have been acquired outside the *Campylobacter* genus and to have spread to it by heterologous genetic exchange. All the resistance determinants from

Campylobacter also confer antibiotic resistance in *E. coli*. Several have been useful in the construction of plasmid vectors (see below).

KANAMYCIN RESISTANCE Three different genetic determinants specifying kanamycin resistance (Km^r) have been identified in *Campylobacter* spp. However, all three act via a similar mechanism, namely the production of a 3'-O-aminoglycoside phosphotransferase. The *Campylobacter*-like organism (CLO) strain BM2196 contains a chromosomally located gene, *aphA-1*, which is almost identical to the Km^r determinant in Tn*903* originally derived from *E. coli* (104). The insertion sequence IS*15*-Δ, which is widespread in gram-negative bacteria, was adjacent to the Km^r gene in BM2196. This result suggests that this Km^r determinant was acquired by *Campylobacter* spp. from a member of the *Enterobacteriaceae* (104).

In contrast, a Km^r determinant from a *C. coli* plasmid pIP1433 of 48 kb (143) specifies a 3'-aminoglycoside phosphotransferase of type III encoded by *aphA-3*, a gene found previously only in gram-positive cocci (148). Therefore, Km^r in *C. coli* could also result from the acquisition of a gene from a gram-positive coccus (71, 148).

A third Km^r phosphotransferase gene, *aphA-7*, was cloned from a 14-kb *C. jejuni* plasmid, pS1178 (144). The DNA sequence of the *aphA-7* gene was most closely related to that in *Streptococcus faecalis;* however, the G+C ratio of the open reading frame of *aphA-7* was 32.8%, which suggested that the *aphA-7* gene may be indigenous to *Campylobacter* (144).

TETRACYCLINE RESISTANCE Tetracycline resistance (Tc^r) determinants from the conjugative *C. coli* plasmid pIP1433 and the conjugative *C. jejuni* plasmids pUA466 and pFKT1025 have been cloned (128, 131, 140, 144), and DNA sequences were determined. Tc^r determinants from both *C. jejuni* and *C. coli* plasmids are highly homologous at the nucleotide level (81, 128, 133) and have been given the designation *tet(O)* to conform to the current nomenclature for Tc^r determinants (75). The *tet(O)* genes demonstrate 75–7[illegible] homology with the *tet(M)* gene of *Streptococcus pneumoniae* (133), and Tet(O) has also been identified in both *Streptococcus* and *Enterococcus* spp. (164), leading to the conclusion that the *tet(O)* determinant has been acquired by *Campylobacter* spp., probably from a gram-positive coccus (128, 133).

The mechanism of Tet(O) resistance remains something of a mystery. The 69-kilodalton (kDa) Tet(O) protein acts at the level of protein synthesis to counteract the inhibitory effects of tetracycline (80). The amino acid sequence of the Tet(O) protein shows considerable homology at the amino terminal end with elongation factor (EF)-Tu and even greater homology with EF-G (80). Homologies extending throughout the entire length of EF-G and Tet(M) (17) and EF-G and Tet(O) (E. K. Manavathu & D. E. Taylor, unpublished data)

have been noted. *Tet(O)* doubtless functions as a GTPase. However, why Tet(O) resembles *EF-G* is not yet clear. Neither EF-Tu nor EF-G have been shown to be *inhibited* directly by tetracycline.

CHLORAMPHENICOL RESISTANCE Sagara et al (120) cloned a resistance determinant specifying chloramphenicol resistance (Cm^r) from a *C. coli* plasmid (*pNR9589*) isolated in Japan. We sequenced the Cm^r determinant and identified a *cat* gene that specified a chloramphenicol acetyltransferase (155). This *cat* gene is most closely related to *cat* genes from *Clostridium perfringens* and *Clostridium difficile* with which it shows 67% identity (4). Because Cm^r is very rare in *Campylobacter* species, *C. coli* may have acquired the *cat* gene from *Clostridium* spp.

Helicobacter Genes

UREASE GENES An unusual feature of all *H. pylori* isolates is the production of a large quantity of urease responsible for hydrolysis of urea to ammonia and carbon dioxide (15). The urease is believed to be an important factor in the colonization by *H. pylori* cells of the gastric mucosa and in their ability to cause damage to mucosal tissue (52, 127). Therefore, genetic studies of *H. pylori* have focused on cloning the genes responsible for urease production.

Clayton et al first reported the cloning of *H. pylori* urease genes using λgt11 and detection of 66-kDa and 31-kDa antigens with antiserum raised against the purified *H. pylori* urease (29). The DNA sequence of the cloned DNA fragment corresponded to two polypeptides, UreA (26.7 kDa) and UreB (60.5 kDa) (28). However, no urease activity was detected in *E. coli* (29).

Using a different strategy, Labigne et al (68) identified a 44-kb portion of the *H. pylori* genome by cosmid cloning that permitted temporary biosynthesis of urease when transferred by conjugation to *C. jejuni*. They took this approach because of the failure of *H. pylori* urease expression in *E. coli*. Subcloning led to localization of the urease gene cluster to a 4.2-kb region of DNA. Four open reading frames in the order *ureC*, *ureD*, *ureA*, *ureB* were identified with predicted molecular weights of 49.2, 15.0, 26.5, and 61.6 kDa, respectively. Only a single copy of the gene cluster is present in *H. pylori*. Polypeptides corresponding to products of *ureA*, *ureB*, and *ureC* but not *ureD* were identified in *E. coli* minicells. The UreA and UreB polypeptides, which correspond to the two structural subunits of urease, appear phylogenetically more closely related to jack bean urease than to other bacterial ureases, which are composed of three subunits. Upstream of both the *ureA* and *ureD* genes (310 bp in each case) is a sequence resembling a σ^{54} promoter (67), which suggests that the expression of *ureA*, *ureB*, and *ureD* genes is under the same transcriptional control and may be subject to nitrogen regulation. In contrast, upstream of the *ureC* gene is an *E. coli* consensus promoter (σ^{70}). The roles of the UreC and UreD polypeptides are not clear. The latter possesses features typical of a transmembrane protein and may function to transport or anchor the enzyme (68). Recently, expression in *E. coli* of urease activity from *H. pylori* was obtained using nitrogen-limitation growth conditions. However, at least four additional genes, designated *ureE*, *ureF*, *ureG*, and *ureH*, cloned from *H. pylori* were required for urease expression in *E. coli* (29a).

M_r 26,000 SURFACE PROTEIN In an unsuccessful attempt (T. J. Trust, personal communication) to purify the fibrillar hemagglutinin (34, 53), O'Toole and coworkers (103) purified a surface protein of unknown function with an apparent M_r of 26,000 from *H. pylori* by extraction with 0.2 M glycine hydrochloride or mild detergent extraction with 0.6% octylglucoside. The N-terminal amino acid sequence for the first 46 residues was determined, and synthetic oligonucleotides capable of encoding amino acid residues 22–26 were synthesized. Those oligonucleotides were used to clone the gene encoding the M_r 26,000 protein within a 900-bp fragment in the vector pK18. Expression of the M_r 26,000 protein in *E. coli* could not be detected in immunoblots but was detected after the fragment was recloned in the expression vector pKK233-2. The size of the protein deduced from the DNA sequence was M_r 22,000. However, the protein produced in *E. coli* and identified using immunoblotting comigrated with that purified from *H. pylori*, i.e. M_r 26,000. The protein produced in *E. coli* appears to be a fusion protein with an additional 45 amino acid residues expressed from within the *E. coli* vector (103). Although several questions remain about the role of this protein in *H. pylori* and its expression in *E. coli*, the gene appears to reside in all *H. pylori* strains tested.

Difficulties Encountered in Cloning and Gene Expression

Examples of the difficulties experienced during the cloning or attempted cloning of *Campylobacter* and *Helicobacter* chromosomal genes are numerous, both in the published literature and in verbal communication. Problems encountered include: failure to express the gene of interest, as for example the *Campylobacter* flagellin genes (48, 99); instability of cloned genes, e.g. the putative *Campylobacter* enterotoxin (20); identification of a peptide of the correct molecular weight but failure to detect enzymic activity, e.g. urease from *H. pylori* (29, 68); and cloning only a portion of the gene, e.g. MOMP from *C. jejuni* (138; K. Hiratsuka & D. E. Taylor, unpublished data) and the M_r 26,000 protein from *H. pylori* (103).

Possible explanations for these difficulties include: (*a*) the presence in *Campylobacter* of unusual promotor sequences that are not recognized or are

recognized much less efficiently in *E. coli;* (*b*) the high A-T content of *C. jejuni* and *C. coli* (32 mol% G+C) and *H. pylori* (36–38 mol% G+C) that may cause DNA sequences from these organisms to be recognized in *E. coli* as strong promotors. Also, regions of 70–80% A-T content that are also rich in static bends can serve as upstream activators of promotors (90). Such strong promotors are stable only in vectors in which efficient terminator signals protect plasmid-control elements from excessive transcription (14). Other possibilities include: (*c*) failure of *E. coli* to process some gene products because of a lack of accessory genes; (*d*) differences in methylation of DNA between *E. coli* and *Campylobacter* that may result in instability; and (*e*) limitation of expression of *Campylobacter* and *Helicobacter* genes due to the presence of suboptimal codons for peptide synthesis in *E. coli*.

First, difficulties related to promotor efficiency can be addressed by using an expression vector, thereby supplying an efficient promotor, rather than relying on the indigenous promotor or on one located within the cloning vector. As DNA sequences of additional *Campylobacter* and *Helicobacter* genes become available, it will be helpful to identify the promoters used and to compare them with those of other bacterial genera. Second, the use of special cloning vectors containing termination signals may be helpful. Such a vector (pJDC9) has been developed for cloning genes from the pneumococcus, which also has a low G+C content, and (as shown by early studies) cannot generate stable DNA fragments greater than 2 kb when cloned in common *E. coli* vectors (27). Third, when lack of accessory proteins for processing presents a problem, one should be able to identify the protein with an antibody, or by its size, without looking for expression directly. Fourth, the use of an *E. coli* strain deficient in methyl-directed DNA repair (*mutL*) may be helpful in cloning some genes. Finally, although some genes from *Campylobacter* specify mRNA with a high percentage of rare codons, these do not necessarily limit expression, at least in the case of *flaA*-specified peptides (100) and the *cat* gene product (155).

PLASMID VECTORS

Shuttle Vectors

The first shuttle vector to be constructed for *E. coli*-to-*Campylobacter* transfer was pILL550, which confered resistance to kanamycin in both *Campylobacter* and *E. coli* (70). This plasmid contains an origin of replication derived from the *C. coli* plasmid pIP445 (70) that functions in *Campylobacter* species as well as one that functions in *E. coli*. The presence of an *oriT* sequence from the IncPα plasmid RK2 (50) enables the vector to be mobilized by a transfer-competent P-group plasmid into *Campylobacter* species. More recently, Wang & Taylor (154) constructed several more shuttle vectors based on a strategy similar to that devised by Labigne-Roussel et al (70). Figure 1 shows

the restriction maps of three shuttle vectors, and Table 2 lists characteristics of these and several additional vectors. All these vectors contain the LacZ' determinant that can complement a defective β-galactosidase in *E. coli* and is useful for selection of clones by their blue and white color on plates containing Xgal (150). Various antibiotic resistance determinants, Cm^r, Km^r, and Tc^r, which consist of the *cat*, *aphA-3*, and *tet(O)* genes, respectively (see section on *Campylobacter* antibiotic-resistance genes), are used as markers for plasmid selection in *Campylobacter*, although all are also expressed in *E. coli*.

Suicide Vectors

Suicide vectors that can be introduced into *Campylobacter* but cannot replicate in these species have also been constructed (69, 153) (see Table 2). This approach has been used to mutagenize 16S rRNA genes (69), to construct a *leuB* mutant of *C. jejuni* by shuttle transposon mutagenesis (A. Labigne, personal communication), and to inactivate flagellar genes in both *C. jejuni* and *C. coli* (47, 156). To be successful, this approach requires that the cloned *Campylobacter* gene be available and that the original copy of the gene be disrupted by insertional mutation, usually with a resistance determinant. Once the suicide vector is mobilized into *C. jejuni*, homologous recombination occurs between the cloned *Campylobacter* gene and the chromosome. In some cells, the original copy of the gene is replaced by the mutated allele to generate the required mutant.

Campylobacter *Vectors*

Plasmids that replicate only in *Campylobacter* have also been constructed (155) (see Table 2). They consist of the origin of replication from a *C. coli* plasmid, a resistance determinant that functions in *Campylobacter* spp., and a

Table 2 *Campylobacter* cloning vectors

Plasmid (size)[a]	Marker[b]	Replication origin		*oriT*[c]	Reference
		E. coli	*Campylobacter*		
pUOA13 (8.7)	*aphA-3, bla, lacZ'*	+	+	+	154
pUOA14 (8.2)	*aphA-3, bla, cat*	+	+	+	155
pUOA15 (11.1)	*bla, lacZ', tet(O)*	+	+	+	154
pUOA17 (8.2)	*aphA-3, lacZ'*	+	+	+	154
pUOA18 (7.4)	*cat*	+	+	+	150
pUOA19 (5.0)	*aphA-3*	−	+	−	150
pUOA20 (4.8)	*cat*	−	+	−	150
pUOA22 (4.1)	*aphA-3, bla*	+	−	−	153
pUOA23 (3.8)	*cat, bla*	+	−	−	153

[a] Plasmid size in kilobases.
[b] The markers *bla* and *lacZ'* are not expressed in *Campylobacter* species.
[c] *oriT* is the origin of transfer from a broad-host-range IncP plasmid (50).

multiple cloning site. These vectors should be useful for subcloning *Campylobacter* DNA fragments into the multiple cloning site with subsequent transfer into *Campylobacter* species by electroporation or natural transformation (see next section). They are being used in our laboratory to search for DNA sequences that enhance uptake of the natural transformation process (154).

GENETIC EXCHANGE MECHANISMS

Plasmid Transfer

CAMPYLOBACTER Conjugative plasmids encoding Cm^r, Km^r, and/or Tc^r are found in some *C. jejuni* but more often in *C. coli* strains (130–132, 134, 135, 145). These plasmids usually range in size from 45 to 50 kb with a G+C content of 31–33 mol%, or approximately equivalent to those of the host species (136). Restriction maps of Tc^r and Km^r plasmids have been constructed (131, 132, 143, 145), but only the resistance determinants have been located on the plasmids. Nothing is known about the arrangements of genes involved in plasmid conjugative transfer or replication. Plasmid transfer frequencies ranged from about 1×10^{-5} to 1×10^{-3} transconjugants per recipient cell in a 24-h mating period. All plasmids tested transferred more efficiently on a solid surface than in liquid medium (135, 136), and their host range was restricted to closely related *Campylobacter* species (130, 135).

HELICOBACTER Plasmid transfer has not been reported in *Helicobacter* species, although plasmids have been visualized in *H. pylori* (108, 147). Tjia

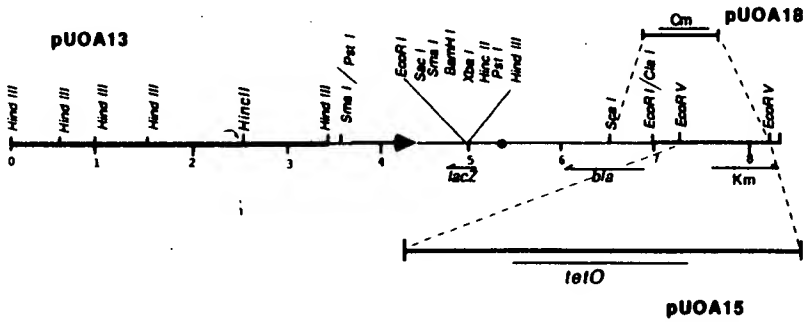

Figure 1 Restriction maps of the shuttle vectors pUOA13, pUOA15, and pUOA18. Position of resistance determinants: Km, kanamycin resistance; Cm, chloramphenicol resistance; *tetO*, tetracycline resistance (all of which are expressed in both *E. coli* and *Campylobacter* species); and *bla*, ampicillin resistance (β lactamase production), which is only expressed in *E. coli*. (*Double bar*) DNA sequence from the Campylobacter plasmid pIP1445; (*arrow*) *oriT* DNA; (*single bar*) pUC13 DNA. Numbers represent kilobase pairs.

et al (147) found that 58% of strains contained one or more plasmids ranging in size from 1.8 kb to 40 kb; whereas Penfold et al (108) found that 48% of strains examined yielded plasmids ranging in size from 3.7 kb to >148 kb. Nucleotide sequence analysis of a 1.5-kb plasmid from *H. pylori* (60a) demonstrated significant homology between it and plasmids that replicated via the "rolling-circle" replication mechanism. This suggests that the plasmid has been acquired by *H. pylori* from a gram-positive coccus since these plasmids have not previously been found in gram-negative bacteria. It has not yet been possible to ascribe a phenotype to any of the plasmids.

No strains of *H. pylori* are as yet reported to be resistant to any of the common antibiotics that are plasmid-mediated in *C. jejuni* and *C. coli*, i.e. Cm^r, Km^r, Tc^r (58, 137). Because these determinants are frequently carried on conjugative plasmids in *Campylobacter* as well as other species, we should not be surprised that conjugative plasmids have not yet been identified in *H. pylori*. Whether or not the increasing use of antibiotics to treat patients with *H. pylori* results in the emergence of plasmid-mediated resistance remains to be seen. However, these organisms, hidden presumably under the mucus layer, have been present in the stomach of patients treated orally with antibiotics for other infections, and have not yet developed resistance. Other factors may be important in the development of resistance, such as the proximity of other bacterial species carrying resistance determinants and the ability of *H. pylori* to take up available DNA.

Natural Transformation

CAMPYLOBACTER Wang & Taylor (154) observed that strains of both *C. coli* and *C. jejuni* could take up DNA without any special treatment, such as $CaCl_2$ or heat shock. Natural-transformation frequencies from streptomycin resistance (Str^r) and nalidixic acid resistance (Nal^r) were approximately 1×10^{-3} transformants per recipient cell for *C. coli* and 1×10^{-4} transformants per recipient cell for *C. jejuni*. Cotransformation frequencies for Str^r and Nal^r were 2×10^{-7} for *C. coli* UA585, which suggests that these two markers are not closely linked. Incubation with DNaseI prevents transformation. Although some strains of *Campylobacter* produce extracellular DNase, there is no association between the DNase-producing ability of the recipient strain and its capacity to take up endogenous DNA. All five *C. coli* strains tested were naturally competent, whereas only three out of six *C. jejuni* strains were competent.

The competence process is not understood in *Campylobacter* species. It is almost completely independent of growth phase; early-log-phase bacteria are slightly more competent than late-log-phase cells. The maximum transformation frequency obtained with *C. coli* UA585, with 0.1 μg DNA/ml, was 4×10^5 transformants per microgram of DNA. DNA from *C. jejuni* UA466R

(Nal^r Str^r) could transform Nal^r to *C. coli* UA585 at about 20% efficiency compared with homologous DNA, but these interspecies Nal^r transformants grew more slowly than either parent. *C. jejuni* UA466 could be transformed to Str^r by *C. coli* UA417R DNA at 1% efficiency; however, these transformants exhibited normal growth rates. Therefore, some cross-species transformation apparently occurs between *C. jejuni* and *C. coli*. Unlabeled *C. jejuni* DNA can also effectively compete with ^{32}P-labeled *C. coli* DNA for uptake into *C. coli* UA585 (154).

Transformation of *Campylobacter* with plasmid DNA is much less efficient than with chromosomal DNA. Small plasmids such as those shown in Table 2 transform *C. coli* UA585 at a frequency 1000-fold lower than that of chromosomal DNA markers. However, when a recipient strain such as *C. coli* UA585 contains a homologous plasmid, transformation frequencies are increased 100-fold. The plasmid present in the recipient seems to act as a rescue plasmid by recombining with the incoming plasmid (154).

If campylobacters are similar to other microorganisms such as *Haemophilus influenzae* and *Neisseria gonorrhoeae*, then their DNA probably contains a specific sequence necessary for binding to and uptake into campylobacter cells. Uptake sequences of 11 base pairs (30) and 10 base pairs (40) have been identified in *H. influenzae* and *N. gonorrhoeae*, respectively. These sequences, which are presumably present throughout the DNA of a particular *Campylobacter* species, may not be present at all on plasmids or perhaps are not present in enough copies to trigger uptake, especially if part of the plasmid has been acquired from an unrelated bacterium that does not possess the relevant uptake sequence.

HELICOBACTER Natural transformation in *H. pylori* has been reported (94). Of 25 clinical isolates, 22 were naturally competent for transformation of Str^r, including the *H. pylori* type strain NCTC 11637. The transformation frequency of *H. pylori* NCTC 11637 was 5×10^{-4}. DNA from a Str^r mutant of *C. jejuni* could not transform competent *H. pylori*. We have confirmed that *H. pylori* strains are naturally competent for transformation. Both Str^r and rifampicin-resistance markers were used to demonstrate DNA uptake (Y. Wang & D. E. Taylor, in preparation).

Both *Campylobacter* and *Helicobacter* spp., therefore, can take up DNA from other individuals in the population. This behavior may be important in the spread of antibiotic resistance such as high level erythromycin and quinolone resistance in *Campylobacter* spp. (33, 43, 142, 163) and perhaps metronidazole resistance in *H. pylori* (6, 39).

Electrotransformation

Electrotransformation, or electroporation, refers to the use of high voltages to induce the uptake of plasmid DNA into cells. Miller et al (86) reported

electroporation of the shuttle vector pILL521 into *C. jejuni* C31 at frequencies as high as 1.2×10^6 transformants per microgram of DNA. Using the commercially available Gene Pulsar apparatus (BioRad), a field strength of 21.5 kV/cm, and a time constant of 2 ms, Yan (160) obtained maximum transformation frequencies of 5×10^3 transformants per microgram of DNA, which was equivalent to approximately 1×10^{-6} transformants per viable cell using *C. jejuni* C31. *Campylobacter* spp. appear to tolerate exposure to high-voltage electric fields without difficulty (86, 160). However, although *C. jejuni* C31 has been used successfully for electroporation, some other strains of *C. jejuni* and *C. coli* do not act as efficient strains in electroporation studies (160).

Bacteriophage Transduction

CAMPYLOBACTER Ritchie et al (117), Grajewski et al (45), and Salama et al (121, 122) have described bacteriophages specific for *C. jejuni* and *C. coli*. These workers have been concerned with developing bacteriophage typing schemes for epidemiological studies of *Campylobacter* infections. None of the Preston phages obtained by Salama and coworkers were lysogenic, and treatment with mitomycin-C was not useful for phage recovery (121). Nevertheless, we have successfully used bacteriophage $\phi 3$ of the Preston typing phages to transduce erythromycin resistance (Ery^r) from *C. coli* UA733 to *C. coli* UA585 (S. Salama & D. E. Taylor, unpublished data). Further studies are required to determine if transduction of the Ery^r marker represents specialized or generalized transduction and to determine if other markers can be transduced by $\phi 3$ or by other *C. jejuni* and *C. coli* bacteriophages.

In an early report, Chang & Ogg described phage-mediated transduction of a Str^r marker between strains of *C. fetus* subsp. *fetus* and from *C. fetus fetus* to *C. fetus* subsp. *venerealis* (23). They were able to effect transduction of glycine tolerance from *C. fetus fetus* to *C. fetus venerealis* using the same phage, VFP-11 (24). These authors pointed out that glycine tolerance may not be a reliable means of separating these two subspecies, as it can be transduced in a single step. Using phage VFP-13, Ogg & Chang (101) demonstrated bacteriophage conversion of a serotype V strain of *C. fetus fetus* to a serotype I strain. Also, some isolates reacted with antiserum to both serotype I and V, showing that both serospecific antigens were produced. Unfortunately, no further work has been published on transduction in these subspecies of *C. fetus*.

HELICOBACTER A single study reports visualization of a bacteriophage with a head size of 70 × 60 nm and a tail of at least 120 nm (123). Lysogeny was maintained during subculture in the laboratory for more than three months. Thus, *H. pylori* may also be capable of bacteriophage transduction.

GENETIC MAPPING

Genome Size and Mapping of Campylobacter *Species*

GENOME SIZES OF *C. JEJUNI* AND *C. COLI* Pulsed-field gel electrophoresis (PFGE) has facilitated the determination of genome sizes and the construction of physical maps of the chromosomes of several bacterial species (66). Bacterial genome sizes vary from *Myxococcus xanthus* (26), the largest, with a genome size of 9.45 Mb, to *Mycoplasma genitalium*, the smallest, with a genome size of only 585 kb (129).

Both *C. jejuni* UA580 (NCTC 1168) and *C. coli* UA417 have genome sizes of approximately 1.7 Mb, as determined using PFGE after *Sal*I and *Sma*I digestion (25). Nuijten et al (97) also determined that *C. jejuni* has a genome size of 1.7 Mb, although others (60) have obtained a slightly higher estimate. Therefore, *Campylobacter* species have genomes that are slightly smaller than *Haemophilus influenzae* Rd at 1.9 Mb (73, 74) and that are only about one-third of the size of the *E. coli* chromosome (126). The small genome size of *Campylobacter* spp. is consistent with their small and delicate nature, requirement for supplemented medium for growth, failure to ferment carbohydrates or degrade complex substances, and their biochemical inertness (57).

GENOME MAPS OF *C. JEJUNI* AND *C. COLI* The genomes of *C. jejuni* UA580 and *C. coli* UA417 consist of a single circular DNA molecule (Figures 2 and 3). One unusual feature of both the *C. jejuni* UA580 and *C. coli* UA417 genome maps is the arrangement of ribosomal RNA genes. Others have demonstrated that *C. jejuni* strains contain three copies of rRNA genes (59, 69, 97). Our data confirm this result, but we found that for both *C. jejuni* UA580 and *C. coli* UA417, at least two of the 16S rRNA genes are located apart from the 23S rRNA genes. Therefore, in the *C. jejuni* and *C. coli* strains we have examined, the rRNA genes do not appear to reside in operons as they do in *E. coli* or *H. influenzae*, but assume an arrangement more characteristic of organisms, such as *Mycoplasma galisepticum*, *Leptospira interrogans*, and *Thermus thermophilus* (66). The *flaAflaB* genes in *C. jejuni* 81116 reside together approximately 300 kb from one 16S rRNA gene and 700 kb from another (97). Similarly, flagellar genes in *C. jejuni* UA580 are located about 700 kb and 170 kb away from the two closest 16S rRNA genes (see Figure 2). These results suggest that the relative locations of flagellar genes and 16S rRNA genes are at least partially conserved in two different *C. jejuni* strains.

Ribosomal protein genes (*rplJ* and *rplL*) that encode, respectively, the large ribosomal subunit proteins L7/L12 and L10 reside together at 90 min on the *E. coli* map (3). A DNA probe from *E. coli* carrying these genes hybridized to two regions in both the *C. jejuni* and *C. coli* maps separated by about 250 kb. The same two regions also hybridized to an *E. coli* probe for the *rpsG* and *rpsL* genes, which encode two small-subunit ribosomal proteins, S7 and S12, and are located at 73 min on the *E. coli* genetic map (3). Therefore, both *C. jejuni* and *C. coli* appear to have two separate clusters comprising a single 16S rRNA gene and both large and small ribosomal protein genes. The Str^r and Ery^r mutations probably also map to ribosomal protein genes (163). However, these genes are not located close to either of the ribosomal gene protein clusters so far identified on the *C. coli* UA417 genome (Figure 3).

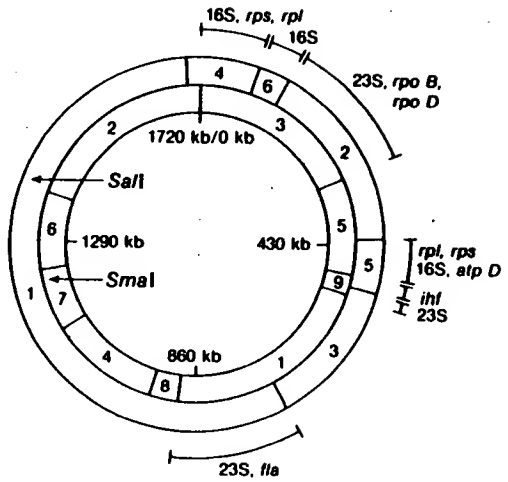

Figure 2 Genome map of *C. jejuni* UA580. Map was constructed from partial digestion patterns obtained with *Sma*I and *Sal*I, and by hybridization of ^{32}P-labeled DNA fragments after extraction from low melting point agarose. Both homologous and heterologous DNA probes were used for mapping. These were *C. jejuni flaAflaB* (pIVB3-300) (99); *C. jejuni* 16S rDNA (pAR140) (115); *E. coli* 23S rDNA (pCW1) (157); *E. coli rplJrplL* (pNO2016) (113); *E. coli rpsGrpsL* (pNO2005) (112); *E. coli hiphimA* (*lhf*) (pPLhip-himA-5) (93); *Bacillus subtilis* RNA polymerase σ^{43} subunit (*rpoD*) (pCP522) (114); *H. influenzae* RNA polymerase β subunit (*rpoB*) (pRIF2) (18); *E. coli atpD* (pBJC505) (151).

Genome Size and Mapping of Helicobacter *Species*

The genome sizes of 30 *H. pylori* strains range from 1.60 to 1.73 Mb (D. E. Taylor, M. Eaton, N. Chang & S. Salama, submitted). The genome of *H. pylori* UA802 is a single circle with a size of 1.71 Mb. *H. mustelae* has

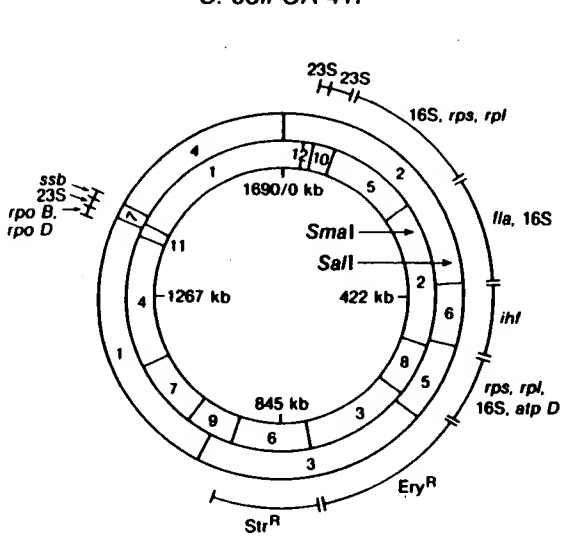

Figure 3 Genome map of *C. coli* UA417. See legend to Figure 2 for map construction and probes used. Additional probe was *E. coli* (*ssb*) (pTL119A 5) (78). Positions of Str^r and Ery^r markers were mapped by natural transformation of extracted DNA fragments (162).

approximately the same genome size as *H. pylori* (D. E. Taylor, unpublished data).

PFGE analysis of *H. pylori* strains from gastric biopsies would indicate that their genomes are highly variable and are far more variable than those of *C. jejuni* or *C. coli*. Yet digest patterns of a single strain maintained in the laboratory remained constant over time, and identical patterns were obtained from three *H. pylori* isolates obtained from antrum, fundus, and body of the stomach of a single patient (D. E. Taylor, M. Eaton, N. Chang & S. Salama, submitted). These results agree with findings from conventional agarose gel electrophoresis of *H. pylori* genome DNA using enzymes with more frequent cut sites (79, 105).

Restriction endonucleases useful for PFGE analysis of *C. jejuni* and *C. coli*, i.e. *SalI* and *SmaI*, are not useful for analysis of *H. pylori*. Instead, *NotI* and *NruI* cut most but not all *H. pylori* strains into a reasonable number of fragments for PFGE analysis. For *H. mustelae*, *SfiI* and *SalI* appear to be the enzymes of choice (D. E. Taylor, unpublished data).

As more genes from *Campylobacter* and *Helicobacter* are cloned and sequenced, it will be useful to extend genome mapping studies of individual

strains to try to determine if gene arrangements are conserved. The amount of repetitive DNA appears to be small within the *C. jejuni*, *C. coli*, and *H. pylori* strains mapped so far because hybridization studies of restriction fragments give clear-cut rather than equivocal results. There is, however, a small degree of variability among the genomes of unrelated isolates of both *C. jejuni* and *C. coli* (161), and this phenomenon is even more pronounced in *H. pylori* (D. E. Taylor, M. Eaton, N. Chang & S. Salama, submitted). Bacterial genomes that constantly undergo genetic exchange via transformation, such as members of *Campylobacter* and *Helicobacter* species, may be prone to rapid genomic rearrangements. Although gene arrangement is usually conserved at the origins and terminus of replication in many bacteria (66), these regions have yet to be identified in *Campylobacter* or *Helicobacter* species.

CONCLUDING REMARKS

The number of investigators interested in the genetics of *Campylobacter* and *Helicobacter* species is increasing. Over the next few years, more genes will be cloned and sequenced from both of these genera. The relative ease with which genes involved in amino acid biosynthesis can be cloned should increase our understanding of these biosynthetic pathways in *Campylobacter* spp. The problems associated with cloning and expression of more specialized *Campylobacter* genes in *E. coli* will probably be overcome by using a variety of different approaches. Unusual features of these organisms that require further investigation include their genome variability, which has been documented in both *C. jejuni* and *C. coli*, but is even more pronounced in *H. pylori*, and the unusual arrangement of their rRNA genes. Studies of regulation of virulence traits in these species should lead to a clearer understanding of the way in which both *Campylobacter* and *Helicobacter* infect their human hosts.

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EXC

5

FAX MESSAGE

Telefax: A DR. ANTONIO [illegible]
Mail: Da DR. ROVINO LACERENZA
Date: 28/7/92 Pag. 1
FAX: 0577-293564

DATE: July 27, 92
FROM: Nuria S./K.F.
CLONTECH
Order/General
FAX #: (415) 424-1352
Technical/Marketing
FAX #: (415) 424-1064

TOTAL # OF PAGES INCLUDING THIS PAGE: 1

Le allego fax ricevuto dalla Clontech
RE:
Attendiamo sue informazioni
REF:

Cordiali Saluti S. Lacerenza

URGENTE!

RE: H. PYROLI GENOMIC LIBRARY

We have attempted by several means to clone the H. pyroli genomic DNA into the EMBL-3 SP6/T7 vector, but the result so far has been negative. We have not been able to come up with an approach that seemed to work better than what we have tried. We are sorry to inform you that we would have to discontinue this custom work until we can come up with an approach that works. However, we are NOT certain when we will be able to come up with a successful solution to this particular cloning problem.

We have used both KW 251 and DH 10B bacterial strains for the cloning. Both strains lack mcrA and mcr B that make cloning of methylated DNA feasible. Unfortunately, even this did not produce the desired results.

We look forward to hearing from you.

CLONTECH

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